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Influence of Polymer Chemistry on Cytokine Secretion from Polarized Macrophages

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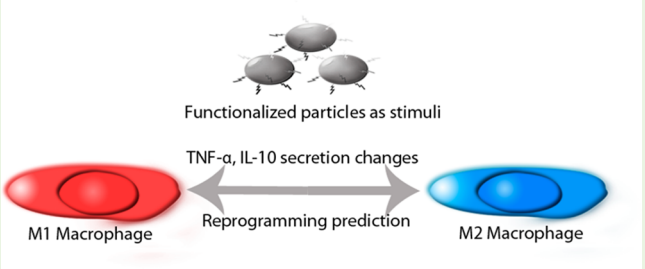
ABSTRACT: Of central importance to tissue engineering and drug delivery is identifying polymer parameters that increase or decrease specific cytokines in response to biomaterials. In this study, we have interrogated the effects of material descriptors and material characteristics on pro-inflammatory, pro-angiogenic, and naïve macrophages using polymeric particles (~600 nm), functionalized with 13 different moieties. We characterized tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) secretion for the three macrophage populations and used the quantitative structure−activity relationship method (QSAR) to accurately predict cytokine secretion for the different macrophage phenotypes. The findings presented here demonstrate that altering cellular responses to polymers can be achieved through exploiting material parameters. For pro-inflammatory macrophages, polarity and the ability to hydrogen bond appear to significantly impact TNF-α secretion while charge impacted pro-angiogenic macrophages. Naïve cells were impacted by charge in a similar manner as the pro-angiogenic cells; however, hydrophilicity also increased TNF-α secretion in these cells. For IL-10 secretion, hydrogen bonding was very negatively correlated with pro-inflammatory cells, whereas it was positively correlated with pro-angiogenic cells.



KEYWORDS: *macrophage phenotypes, cytokines, TNF-α, IL-10, polymer properties*

1. INTRODUCTION

Surfaces play an integral role on cellular responses.[1−4] The properties that these surfaces possess, such as hydrophobicity[5,6] and stiffness,[7−9] can influence the cell, in particular the cytokines secreted.[10−12] Altering the chemical properties of the polymers that interact with cells can modulate their activity.[13] In particular, these surfaces can alter macrophage phenotype. Macrophages exist on a continuum of phenotypes with the extremes being pro-inflammatory, known as M1 macrophages, and pro-angiogenic, referred to as M2. The classically activated M1 phenotype arises in response to interferon-γ (IFN-γ) or lipopolysaccharide (LPS).[14] The pro-angiogenic alternatively activated M2 macrophages are generated upon exposure to interleukine-4 (IL-4).[15,16] Alternatively activated macrophages have been shown to reduce histological and functional injury as opposed to classically activated macrophages,[17] and thus, developing an understanding of the material properties that promote M2 macrophages would be a useful tool in tissue engineering and for the field of biomaterials.

A large amount of these pro-inflammatory mediators are secreted by macrophages and help tumor cells proliferate and survive. A consequence of this was that normal cytokine secretion was suppressed. These early tumor cells were called M1-like macrophages, which are transformed to M2-like macrophages.[18] These cells, also called tumor-associated macrophages (TAMs), produce a large amount of IL-10, express a low level of pro-inflammatory cytokines, and possess poor antigen presenting capabilities. Specifically, TAMs produce anti-inflammatory cytokines such as IL-10 and angiogenic factors such as transforming growth factor, epidermal growth factor, and vascular endothelial growth factor. They also secrete a decreased level of reactive nitrogen intermediates and have decreased antigen presentation and tumoricidal capacity, as measured by a decrease in tumor necrosis factor-α (TNF-α). The expression profile of TAMs drives cellular proliferation and angiogenesis.[19−21] Reprogramming tumor-promoting macrophages is a potential therapeutic strategy that has been addressed through administration of IL-12.[22] IL-12 enhances antigen presentation in macrophages and can induce IFN-γ, which promotes an M1 phenotype.[23] The potential for polymers to alter cytokine secretion has been demonstrated,[13] illustrating the need to develop predictive models for these cellular responses.

Here, we present a potential platform for engineering macrophage responses to elicit changes in cytokine secretion for possible use as a drug delivery vehicle for chemotherapeutics that reprograms TAMs to act synergistically with the drug regimen. In particular, this study focuses on increasing the level of TNF-α and decreasing the level of IL-10 expressed

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by macrophages. TNF-α is toxic to tumor cells at sufficient levels[24] and tumor promoting at others.[25] Furthermore, the maximum tolerated dose for systemic delivery of TNF-α in humans is 200−400 mg/m^2,[26,27] which is too low to induce a response in tumors. IL-10 is a pleiotropic cytokine that can activate and induce proliferation of B-lymphocytes and confer survival to certain tumors.[28,29] The preceding citations and related discussion illustrate the importance of altering macrophage cytokine production profiles at the tumor site to cause tumor regression. In this paper we demonstrate the ability to alter TNF-α and IL-10 through modifying surface properties of a model polymeric particle for potential applications in immunomodulative drug delivery. The library of materials examined were based on p(*N*-isopropylacrylamide-*co*-acrylic acid) (p(NIPAm-*co*-AAc)), which has good biocompatibility and has attracted attention from both academic and industrial researchers.[30−33] The macrophages were treated with LPS or IL-4 to induce M1 or M2 phenotypes. Naïve cells were also tested. Correlations between the cytokines secreted and the material properties were examined and a predictive mathematical model for the two cytokines and three macrophage treatments was developed.

2. MATERIALS AND METHODS

Thirteen chemically unique functional groups were coupled to polymers to assess how they affect macrophage activation. All experiments had at least four replicates and error bars indicated the standard deviation. All materials were purchased from Sigma (St. Louis, MO) and used as received, unless otherwise indicated. Fresh deionized water (Milli-Q, Barnstead Nanopure, Thermo Scientific, Waltham, MA) was used throughout this study.

Monomers that were coupled to p(NIPAm-*co*-AAc) particles were: 3-butenylamine (Santa Cruz Biotechnology, Dallas, TX);1,4-dioxan-2-ylmethanamine; glycidamide; 4-amino-3-penten-2-one; malonamide (Fisher, Pittsburgh, PA); *tert*-butyl 4-aminobutanoate (VWR, Radnor, PA); aminoacetaldehyde dimethyl acetal (Alfa Aesar, Ward Hill, MA); 3-aminobenzamide oxime; 2,4-dinitro-phenyl-hydroxylamine; 1-Amino-4-oxocyclohexanecarboxylic acid ethylene ketal; 2-amino-ethylmethylsulfone hydrochloride; 3-amino-1-propanesulfonic acid (Fisher, Pittsburgh, PA); and aminomethylphosphonic acid (Alfa Aesar, Ward Hill, MA).

2.1. Polymer Synthesis. *2.1.1. p(NIPAm-co-AAc) Particle Synthesis.* The synthesis and modification of p(NIPAm-*co*-AAc) particles has been described previously.[34] Briefly, NIPAm (2.4 g), *N*,*N*-methylenebis(acrylamide) (0.16 g), and 157 μL of AAc (J.T. Baker, Center Valley, PA) were dissolved in 100 mL of H_2O and stirred under N_2 in a 250 mL round-bottom flask for 30 min at 70 °C. Then, 200 mg of $K_2S_2O_8$ was dissolved in 10 mL of H_2O and added to the flask. After 4 h, the suspension was slowly cooled to room temperature, filtered with P5 grade filter paper, and dialyzed for 48 h in Milli-Q water. The particles were freeze-dried using a lyophilizer (Labconco, Kansas City, MO, 4.5L).

In a 15 mL tube, 3 mL of phosphate buffered saline (PBS, diluted from 10X solution, Fisher Scientific to 0.1M, pH 7.4), 6 mg of monomers, 0.6 mL of 5% w/v p(NIPAm-*co*-AAc) particles, and 60 mg of 1-ethyl-3(−3-(dimethylamino)propyl) carbodiimide hydrochloride were vortexed and incubated overnight at room temperature. The particles were dialyzed for 24 h in H_2O and lyophilized. The particles were reconstituted at 1% w/v in H_2O. To ensure that the modifications proceeded to the same % reaction, NMR was used to characterize the modified particles. The modified particles were suspended in D_2O. The ^{1}H spectra were recorded on a Bruker Avance III Spectrometer with a sweep width of 6602.1 Hz, a 90° pulse, and an acquisition time of 2.48 s. Sixteen repetitive scans with 64k points were acquired and the data were processed in MNova with 128k points, zero filling, and exponential line broadening of 1.0 Hz. NMR was able to determine that the 54 ± 2% of carboxylic group on AAc were modified for all of the functionalizations.

2.2. Cell Viability. RAW 264.7 macrophages were cultured at 37 °C with 5% CO_2 in 10% fetal bovine serum, 100 U/L penicillin, and 100 μg/L streptomycin in Dulbecco's modified Eagle's medium (DMEM High Glucose; Thermo Scientific), to be referred to as complete media (CM). RAW 264.7 cells were seeded in a 24-well plate at 1.25 × 10^5 cell/cm^2 in the presence of 5 μg/mL LPS or 25 ng/mL IL-4 (eBioscience Inc., San Diego, CA) and incubated 24 h at 37 °C in 5% CO_2. A control set of experiments was not activated. After activation, particles were added to the wells (100 particles per cell) and incubated for 24 h. All particles were sterilized by washing three times in 70% ethanol, followed by three washed in sterile H_2O by centrifuging at 10 000 g for 3 min. In a control experiment, cells were incubated without particles. A control of particle and IL-4 or LPS in the absence of cells was also conducted. The media in each well was collected and stored at −20 °C.

Cell viability was determined through an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. After the supernatants were removed, 50 μL of a MTT solution (5 mg/mL in DI water) and 500 μL of CM were added to the wells and incubated for 2 h at 37 °C in 5% CO_2. Then 425 μL of media was aspirated. The crystalline deposits were dissolved using 500 μL of dimethyl sulfoxide (DMSO, Fisher, Pittsburgh, PA). The optical density (OD) at 540 nm was measured using a plate reader (BioTek Synergy HT Multi-detection Microplate Reader, Winooski, VT) with a reference at 690 nm.

2.3. Measurement of Cytokine Production. The supernatants removed in the above section were analyzed for cytokine production. Secretion of tumor necrosis factor-α (TNF-α) and IL-10 in LPS, IL-4, or naïve cell supernatant were determined by commercially available immunoassay kits (eBioscience Inc., San Diego, CA) and performed as described by the manufacturer.

2.4. Statistics and Data Analysis. Statistical analysis was performed using XLSTAT statistical software (New York, NY). Statistical significance of the mean comparisons was determined by ANOVA. Differences were considered statistically significant for $p < 0.05$. The data generated for IL-10 and TNF-α secretion in LPS, IL-4, and naïve cells were split into two sets: training and validation sets, making sure that the values in the validation set were distributed throughout the range of secretions. The training set was used to develop the QSAR model through partial least-squares regression. The materials descriptors (Table 1) were developed based on descriptors defined by Bicerano.[35] The validation set was used to verify the QSAR model. The validation sets for TNF-α were: alkene, ether, ketone, amide, and phosphonic acid for LPS stimulated cells; epoxide, ester, ketone, nitro, sulfone, and unmodified for IL-4 stimulate macrophages; and acetal, alkene, ether, sulfonic acid, and unmodified for naïve cells. The validation sets for IL-10 were: acetal, amide, epoxide, ketal, and sulfonic acid for LPS stimulated cells and acetal, amide, ether, nitro, and phosphonic acid for IL-4 stimulate cells. Because the secreted protein was not well correlated between stimulations or surface modifications, the validation sets were chosen independently for each cytokine and activation.

3. RESULTS

3.1. Cell Viability. Functionalized p(NIPAm-*co*-AAc) particles were synthesized, functionalized, and characterized for their ζ-potential, water contact angle (WCA), melting temperature, and ability to activate complement through the alternative pathway as previously described.[34] The chemical structures of the molecules attached to the particles are shown in Figure 1. These results have been reprinted with permission[34] in Figure 2. The particles size was confirmed through scanning electron microscopy and the % functionalization of the particles was analyzed through NMR. Cell compatibility studies of these materials were performed by treating RAW 264.7 macrophages with LPS or IL-4 for 24 h,

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Table 1. Twenty-Two Molecular Descriptors for the Surface Modifications Used in This Study[a]

ID	structure descriptor	descriptions
1	R	freely rotating bonds
2	H_D	H-bond donors
3	H_A	H-bond acceptors
4	N_{sp2}	number of sp^2 carbon atoms
5	$N_{1\ °C}$	number of 1° carbon atoms
6	^1X	connectivity index 1
7	$^1X^v$	connectivity index 2
8	^0X	atomic index 1
9	$^0X^v$	atomic index 2
10	N	number of non-hydrogen atoms
11	N_C	number of carbon atoms
12	N_H	number of hydrogen atoms
13	N_O	number of oxygen atoms
14	N_N	number of nitrogen atoms
15	N_{CH2}	number of CH_2
16	N_K	$N_K = 5N_{amide} + 4N_{hydroxyl} - 3N_{ether} - 5N_{C=C} + 3N_{sulfone}$
17	N_{dc}	$N_{dc} = 19N_N + 12N_{(side\ group\ O,-S-)} + 52N_{sulfone} - 14N_{cyc}$
18	N_{group}	$N_{group} = 12N_{hydroxyl} + 12N_{amide} + 2N_{(nonamide\text{-}(NH)\text{-}unit)} - N_{(alkyl\ ether\text{-}O-)} - N_{C=C} + 4N_{(nonamide\text{-}(C=O)\text{-}next\ to\ a\ nitrogen)} + 7N_{(-(C=O)\text{-}incarboxylic\ acid,\ ketone,\ or\ aldehyde)} + 2N_{(other-(C=O)-)}$
19	M	molecular weight
20	T_{am}	melting temperature
21	ζ	zeta potential
22	WCA	water contact angle

[a]The structure descriptors are defined by Bicerano.[34].

followed by addition of functionalized p(NIPAm-*co*-AAc) particles and assayed for metabolic activity 24 h later. Naïve macrophages were also tested. The results for the cytotoxicity experiments have been previously reported.[34] The p(NIPAm-*co*-AAc) particles showed minimal toxicity for all treatments and for all particles. RAW 264.7 cells are a common model for studying macrophage function and activation.[36,37]

3.2. TNF-α and IL-10 Secretion from Activated and Naïve Macrophages. TNF-α production by activated and naïve macrophages in response to 24h incubation with functionalized p(NIPAm-*co*-AAc) particles is shown in Figure 3A. Functionalizations acetal, alkene, ester, ether, ketal, ketone, nitro, oxime, phosphonic acid, and sulfonic acid all decreased TNF-α expression compared to the unmodified p(NIPAm-*co*-AAc) particles ($p < 0.05$) for LPS stimulated macrophages. The largest decrease was ~40% for the oxime functionalized particle. A control of LPS stimulated macrophages without particles was also run and yielded 4.89 ± 0.59 ng TNF-α /250 000 cells, which was statistically higher than cells treated with alkene, ketal, ketone, nitro, or oxime functionalized particles ($p < 0.05$). For IL-4-treated macrophages, ester, ether, ketal, and sulfonic acid functionalized particles resulted in statistically larger TNF-α expression compared to the unmodified particles ($p < 0.05$), whereas amide, nitro, and oxime decreased TNF-α. Cells treated with IL-4 and no particles resulted in 1.55 ± 0.22 ng TNF-α/250 000 cells. In comparing to cells treated with IL-4 without particles, ether, ketal, and sulfonic acid functionalized particles increased TNF-α secretion ($p < 0.05$). The naïve macrophages without particles yielded 0.55 ± 0.02 ng TNF-α /250 000 cells. With the exception of the unmodified p(NIPAm-*co*-AAc) and oxime, phosphonic acid, and sulfone modified particles, the addition of particles resulted in a significant increase in TNF-α. Sulfonic acid and ketal functionalized particles resulted in a 15-fold increase over unmodified particles. Acetal, epoxide, ester, ether, and nitro functionalized particles increased TNF-α expression 7-fold, and alkene, amide, and ketone increased secretion 3-fold.

IL-10 secretion for LPS and IL-4 stimulated macrophages is shown in Figure 3B. IL-10 secretion for naïve cells is also given in Figure 3B as 10× the secreted ng IL-10/250 000 cells. LPS resulted in no significant differences between the unmodified p(NIPAm-*co*-AAc) particles and the control without particles, which secreted 4.33 ± 0.72 ng IL-10/250 000 cells. Statistically

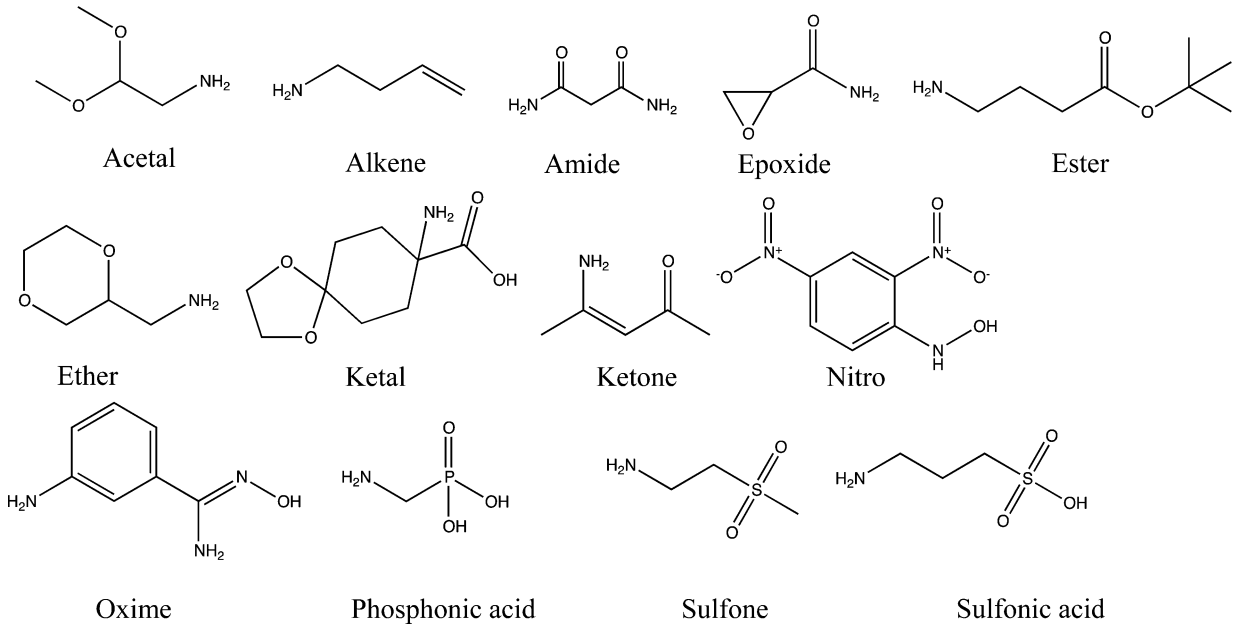

Figure 1. Chemical structures of all molecules used for the modification of p(NIPAm-*co*-AAc) particles through the amine group. Their functional groups were used as labels in following figures for convenience.

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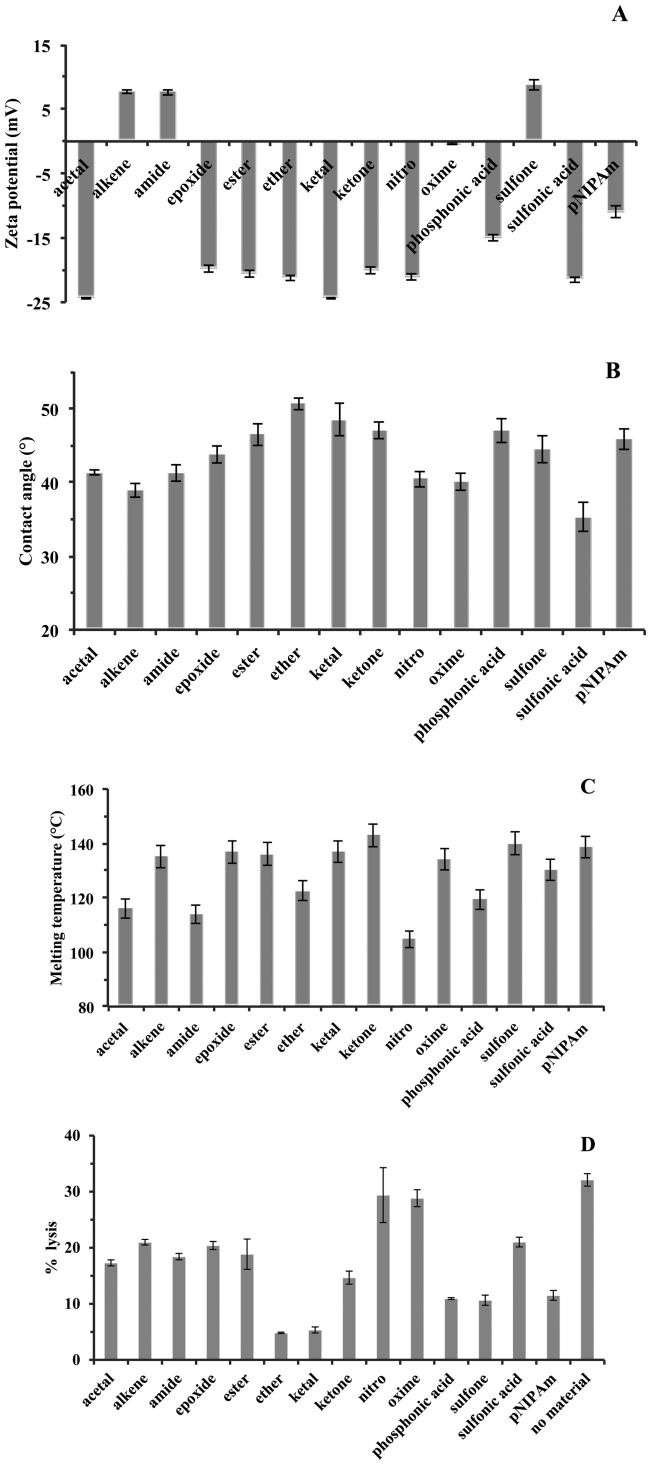

Figure 2. Material characterization of modified p(NIPAm-*co*-AAc) particles. (A) Zeta potential, (B) water contact angle, (C) melting temperature, and (D) percentage of red blood cell lysis from alternative activation of complement. Zeta potential and percentage of lysis data represents three replicates for each sample. Water contact angle represents five replicates for each sample. All data are shown by mean value ± standard deviation. Reproduced with permission.[34] Copyright 2014 American Chemical Society.

significant decreases in IL-10 were observed for ether, oxime, phosphonic acid, and sulfonic acid functionalized particles ($p < 0.05$). A greater variation in expression levels was observed for cells stimulated with IL-4 in the presence of particles. All of the modified polymers except oxime were statistically higher than the unmodified particle, with ketal being 5-fold and sulfonic acid being 3-fold higher than the unmodified particle. The IL-4 stimulated control without particles secreted 2.31 ± 0.06 ng IL-10/250 000 cells. The naïve cells secreted very low levels of IL-10 with the control without particles yielding 0.16 ± 0.01 ng IL-10/250 000 cells. The expression levels shown in Figure 3B for the naïve cells are 10× higher than the measured values. The limit of detection and limit of quantification were found to be 0.03 and 0.07 ng IL-10/250 000 cells, indicating that all samples had detectable IL-10, but not all were quantifiable. For this reason, statistical differences between naïve macrophages treated with particles were not considered.

4. DISCUSSION

4.1. Material Parameters That Influence TNF-α Secretion in Activated and Naïve Macrophages.

Secretion of TNF-α in the absence of particles was highest for LPS-treated, followed by IL-4-treated, and naïve cells, which is expected.[13,36,38] Upon addition of particles, LPS-treated particles secrete more TNF-α than their IL-4-treated counterparts. The naïve cells, however, express significantly more TNF-α than their LPS-treated counterparts for ester, ether, ketal, nitro, and sulfonic acid functionalized particles ($p < 0.05$). One of the factors that can cause the naïve macrophages to produce more TNF-α than the LPS stimulated cells is that IL-10 is a suppressor of TNF.[39] Nathan and co-workers have reported that 0.1 ng/mL IL-10 could suppress TNF-α secretion from LPS stimulated cells by a factor 21.4 ± 2.5.[40] An additional explanation is that macrophages can develop an LPS tolerance in which synthesis of TNF-α is diminished.[41]

The focus of this work was to examine how material properties influence cytokine expression from activated and naïve macrophages. Partial least-squares analysis was performed on the TNF-α secretion from LPS, Il-4, and naïve macrophages and the material properties of the particles incubated with those macrophages are shown in Figure 4. QSAR was used to build equations connecting the material descriptors in Table 1 to the TNF-α expression. The data set was split into a training set, which was used to build the mathematical model, and a validation set, which measured the predictive ability of the model. The model was able to develop equations for TNF-α expression that were dependent on six or fewer material parameters. The models are shown below.

$$\mathrm{TNF}-\alpha_{\mathrm{LPS}} = 5.387 + 0.063N_{\mathrm{K}} - 0.067{}^{0}\mathrm{X}^{\mathrm{v}} - 0.163H_{\mathrm{D}} - 0.016\mathrm{H}_{\mathrm{A}} - 0.060N_{\mathrm{sp2}} \quad (1a)$$

$$\mathrm{TNF}-\alpha_{\mathrm{IL-4}} = 1.502 - 0.019\zeta + 0.142N_{\mathrm{CH2}} - 0.063{}^{1}\mathrm{X} + 0.225{}^{1}\mathrm{X}^{\mathrm{v}} - 0.153\mathrm{H}_{\mathrm{A}} \quad (1b)$$

$$\mathrm{TNF}-\alpha_{\mathrm{naïve}} = 17.196 - 0.155\zeta - 0.392\mathrm{WCA} + 0.140N_{\mathrm{group}} + 1.473N_{\mathrm{CH2}} + 0.505N_{\mathrm{O}} - 0.832\mathrm{H}_{\mathrm{D}} \quad (1c)$$

The results for the goodness of the fits on the training set for the above equations are as follows: (1a) the predicted *Y*-variation, $Q^2Y = 0.931$ and the explained *Y*-variation, $R^2Y = 0.980$; (1b) $Q^2Y = 0.974$, $R^2Y = 0.995$; and (1c) $Q^2Y = 0.843$ and $R^2Y = 0.975$ (Figure 4). For a good fit, the difference between R^2Y and Q^2Y should be less than 0.2,[42,43] with a

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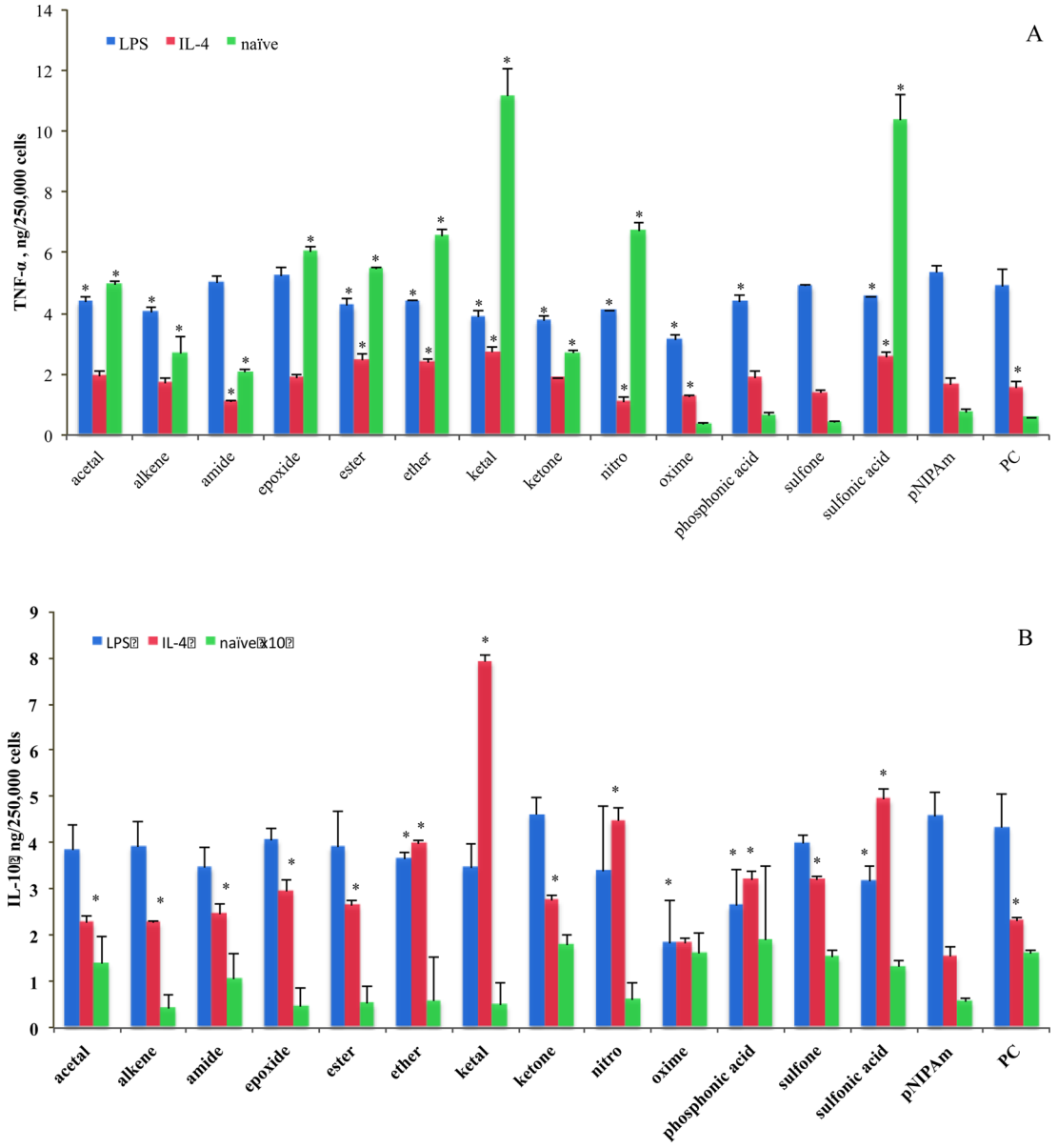

Figure 3. Cytokine expression (A) TNF-α and (B) IL-10 secretion by macrophages in response to functionalized p(NIPAm-*co*-AAc) particles and stimulation with LPS or IL-4. Naïve cells are also shown. Positive controls (PC) without particles are shown for both activations, as well as for naïve cells. * $p < 0.05$ compared with pNIPAm.

difference greater than 0.2 indicating the presence of outliers. Eriksson has stated that $Q^2Y > 0.5$ is generally regarded as good and $Q^2Y > 0.9$ is excellent.[42] For in vitro experiments, R^2 should be ≥0.81 for a good fit.[44] Our models were able to satisfy these requirements.

For M1 macrophages there was a positive correlation (R = 0.818) with N_K and a negative correlation with N_{sp2} (R = −0.719). N_K is used as a correction factor for the prediction of viscosity and includes polar functional groups, along with C═C. In addition, hydrogen bond donors and acceptors have negative correlations with TNF-α expression (R = −0.836 and −0.532, respectively). The atomic index 2, which is a measure of the hybridization and number of hydrogen atoms attached to each atom in the modification listed in Figure 1, with fewer hydrogen atoms and increased hybridization leading to a larger number. It is important to note that the number of hydrogen atoms attached to non-hydrogen atoms in the modification is generally more impactful than the hybridization on the atomic index. The correlation between TNF-α expression and the atomic index 2 is R = −0.698. The overarching theme of these parameters appears to be that the polarity and ability to H-bond alters the TNF-α expression for M1 polarized cells.

For M2 polarized cells, there are positive correlations with the number of CH_2 groups; and the first and second connectivity indices (R = 0.819, 0.574, 0.733, respectively) and a negative correlation with the ζ-potential and hydrogen bond acceptors (R = −0.843 and −0.248). Taken together, these factors indicate that the charge of the particle is influential on the TNF-α expression for M2 polarized cells.

Naïve cells were exposed to modified particles and their TNF-α secretion was modeled through QSAR, which is shown in eq 1c. Six parameters were used to build the model including ζ-potential (R = −0.714), WCA (R = −0.320), N_{group} (R = −0.182), the number of CH_2 groups (R = 0.660), the number

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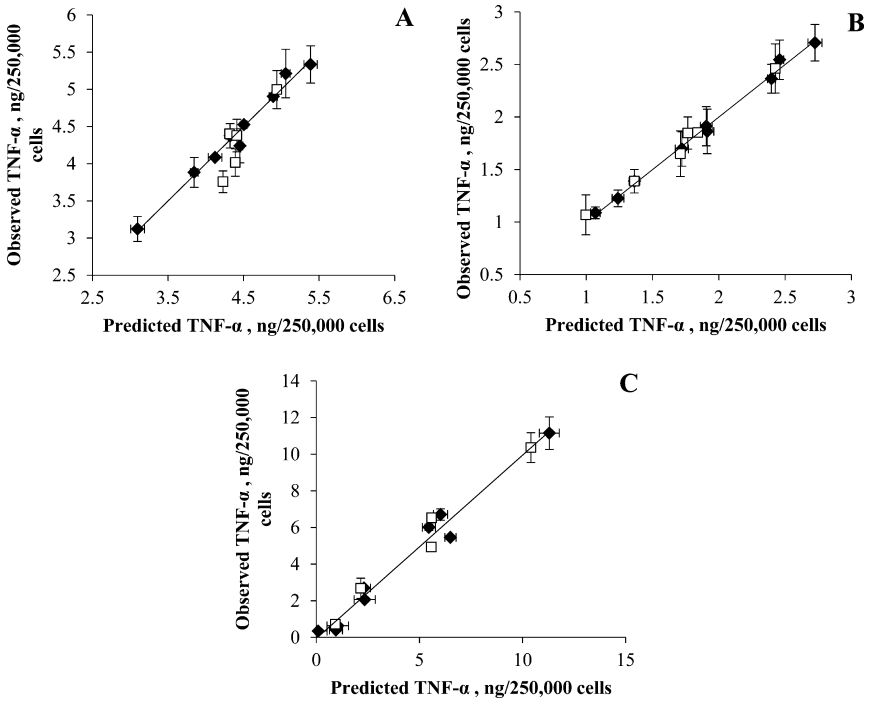

Figure 4. Plot of experimentally determined (observed) versus predicted values for TNF-α secretion. Error bars represent the standard deviations of the observed and predicted values. The solid symbols are the training set and the open symbols represent the validation set. (A) LPS-treated cells. The results for the statistics of the model were the following: $Q^2Y = 0.931$ and $R^2Y = 0.980$. (B) IL-4-treated cells. The results for the statistics of the model were the following: $Q^2Y = 0.974$ and $R^2Y = 0.995$. (C) Naïve cells. The results for the statistics of the model were the following: $Q^2Y = 0.843$ and $R^2Y = 0.975$.

of oxygen atoms ($R = 0.634$), and the number of hydrogen bond donors (R = −0.407). These parameters suggest that hydrophilic, negatively charged particles will increase the amount of TNF-α secreted from the cells. In examining Figure 3A ketal and sulfonic acid functionalized particles secrete substantially more TNF-α compared to the other functionalized particles incubated with naïve cells and with all of the LPS and IL-4 stimulated macrophages. In examining material properties such as ζ-potential and WCA, neither ketal nor sulfonic acid functionalized particles exhibited extreme behaviors. The same can be said for the other parameters listed above that were used in constructing the model. This demonstrates how multiple material parameters and characteristics influence cell behavior. Take, for example, the number of CH_2 groups present in the molecules. The ketal and sulfonic acid modifiers have six and three CH_2 groups respectively, whereas the ether modifier has four CH_2 groups but secretes approximately half the TNF-α of the other two particles. This results from the ether functionalized particles having a lower N_{group} and fewer oxygen atoms present in the modifier.

In examining all of the material parameters that influence TNF-α expression for LPS and IL-4 stimulated cells, along with the naïve population, there are several commonalities that appear. The first of which is the number of hydrogen bond donors and hydrogen bond acceptors, which appear in all three equations: both LPS stimulated and naïve cells have negative correlations with the number of hydrogen bond donors while IL-4 and LPS stimulated macrophages have negative correlations with the number of hydrogen bond acceptors. This may be explained by an increase in protein adsorption on the surface of the particle, which results in an increased response to the particle.[45] In a previous study we measured the alternative activation of complement through a red blood cell lysis assay in which the p(NIPAm-*co*-AAc) particles were incubated with C4-deficient guinea pig serum. The serum was separated from the particles, exposed to red blood cells, and the amount of hemoglobin present in the supernatant was used to measure the complement activation through the alternative pathway.[34] It is important to note that materials that activate complement would deplete complement from the serum and result in a lower measured % lysis. The amount of TNF-α secreted from LPS and IL-4 stimulated cells negatively correlated with the % lysis of red blood cells ($R = -0.414$ and −0.524), meaning that activating complement resulted in increased TNF-α. The correlation for naïve cells and complement was −0.167, indicating that there is likely another explanation for the naïve cells. Another commonality in eqs 1b and 1c is surface charge. In both of these equations a negatively charged particle increases the amount of TNF-α. N_{group} in eq 1c and N_K in eq 1a are also reflections of the charge or polarity of the particle. Previous research has shown that TNF-α secretion is negatively correlated with surface charge.[46,47]

Although there are similarities in the equations generated for activated and naïve macrophages, it is important to note that different macrophage states respond differently to the modifications presented in Figure 1. The correlations for TNF-α secretion for LPS activated with IL-4 activated or naïve cells are very low ($R = -0.084$ and −0.133). IL-4 stimulated cells and naïve macrophages are better correlated ($R = 0.689$). The lack of correlation between LPS activated cells and the other states may be a result of endotoxin tolerance.

4.2. Material Parameters That Influence Il-10 Secretion in Activated Macrophages. Secretion of IL-10 in the absence of particles was highest for LPS treated, followed by IL-4-treated, and naïve cells. LPS priming has been shown to

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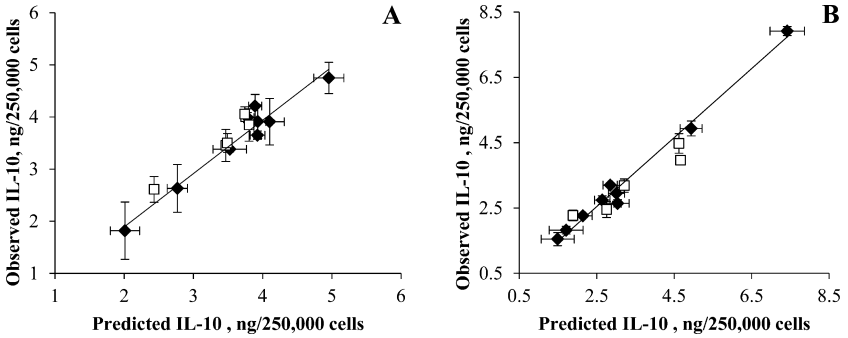

Figure 5. Plot of experimentally determined (observed) versus predicted values for IL-10 secretion. Error bars represent the standard deviations of the observed and predicted values. The solid symbols are the training set and the open symbols represent the validation set. (A) LPS treated cells. The results for the statistics of the model were the following: $Q^2Y = 0.849$ and $R^2Y = 0.948$. (B) IL-4-treated cells. The results for the statistics of the model were the following: $Q^2Y = 0.811$ and $R^2Y = 0.942$.

induce IL-10 secretion.[48−50] Additionally, IL-4-treated macrophages are characterized by high IL-10 secretion.[51]

To determine how material properties influence IL-10 secretion from LPS- and IL-4-activated macrophages, partial least-squares analysis was performed on the data set in Figure 3B and the following equations were developed using the material descriptors in Table 1.

$$\mathrm{IL-10_{LPS}} = 4.956 - 0.013{}^{1}\mathrm{X} + 0.150{}^{1}\mathrm{X}^{v} - 0.607\mathrm{H_D} - 0.090\mathrm{H_A} \quad (2a)$$

$$\mathrm{IL-10_{IL-4}} = 1.491 - 0.236\mathrm{H_A} + 0.344{}^{1}\mathrm{X} + 0.108N - 0.237N_{\mathrm{H}} + 0.487N_{\mathrm{O}} + 0.823N_{\mathrm{CH2}} \quad (2b)$$

The results for the goodness of the fits on the training set for the above equations are as follows: (2a) $Q^2Y = 0.849$, $R^2Y = 0.948$ and (2b) $Q^2Y = 0.811$, $R^2Y = 0.942$ (Figure 5). Because seven of the particles incubated with naïve cells resulted in IL-10 secretion below the limit of quantification, QSAR was not performed on the naïve samples. As with the previous cytokine, a training set (closed symbols) was used to develop the mathematical model and a validation set (open symbols) was used to verify the predictive nature of the model.

For M1 macrophages there are negative correlations with connectivity indices 1 and 2; the number of hydrogen bond donors; and the number of hydrogen bond acceptors (R = −0.547, −0.584, −0.957, −0.505). Lysis of red blood cells had a modest negative correlation with IL-10 (R = −0.355). Because the correlation between IL-10 secretion and hydrogen bond donors is quite strong, there may be protein interactions with the particle other than complement that are contributing to this strong correlation. In comparing the material parameters for IL-10 secretion with those for TNF-α for the LPS treated cells, the negative correlations for both equations with hydrogen bond donors and acceptors is immediately obvious. The correlation between these two cytokines is R = 0.517, which further indicates that they may be released through related interactions with the particles, which is consistent with previous research indicating that IL-10 negatively regulates TNF-α.[39,49,52]

Unlike the LPS treated macrophages, IL-4 macrophages exhibited positive correlations with all of the parameters used to develop the mathematical expression in eq 2b (hydrogen bond acceptors, R = 0.632; connectivity index 1, R = 0.706; the number of non-hydrogen atoms, R = 0.622; the number of hydrogen atoms, R = 0.516; the number of oxygen atoms, R = 0.875; and the number of CH_2 groups, R = 0.883). The difference in dependence on polymer parameters for the two activations can further be seen in the lack of a correlation between the IL-10 secreted for LPS- and IL-4-treated cells (R = 0.128).

Similar to the LPS-stimulated cells, the IL-4-treated cells had a correlation of R = 0.543 between IL-10 and TNF-α secretion. In comparing the two equations, the correlation between the two cytokines and the number of CH_2 groups is quite strong. Because hydrogen bonding is present in eqs 1b and 2b, it is likely that how proteins interact with the particle changes how the cell responds. Protein adsorption to biomaterials is a very active field of research in biomedical engineering. Increasing the hydrogen bonding character of a polymer is generally correlated with excellent blood compatibility,[53,54] because of the ability of the polymer to interact well with water, thus permitting the protein to retain its tertiary structure.[55] eqs 2a and 2b show that increasing the amount of hydrogen bonding will decrease the IL-10 secretion for both LPS and IL-4 stimulated cells, thus indicating that polymers that interact poorly with water will increase the secretion of IL-10. As with the M1 cells, there was a low correlation with % lysis (R = −0.337) suggesting that although a complement may be interacting with the particle, causing IL-10 to be secreted, there is likely another protein or protein system that is responsible for altering IL-10 secretion in IL-4-treated cells.

5. CONCLUSIONS

In summary, we have developed mathematical equations that have been able to predict TNF-α and IL-10 secretion for LPS- and IL-4-stimulated cells. TNF-α secretion was also predicted for naïve cells. All of the equations had a dependence on hydrogen bond donors and/or hydrogen bond acceptors. Despite this commonality, there are differences between the equations generated that allow for the possibility of reprogramming cells, or altering the amounts of specific cytokines secreted, using polymeric particles without delivery of a protein. The generalities uncovered in this work pave the way for altering cellular responses for applications in drug delivery and tissue engineering.

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Notes

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